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**Fractionation of milk proteins, especially of milk produced on protein-free feed with urea and ammonium salt as the sole source of nitrogen**

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# FRACTIONATION OF MILK PROTEINS, ESPECIALLY OF MILK PRODUCED ON PROTEIN-FREE FEED WITH UREA AND AMMONIUM SALT AS THE SOLE SOURCE OF NITROGEN

BY

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(with 4 figs.)

(9—VIII—1965)

HANS VON EULER was interested in problems of a most varied nature during his long and to the very end of his busy life. One of his interests was in nutritional problems which he studied experimentally during a period of many decades to the end of his life. Also his wife BETH took part effectively in these studies. When I considered the subject of my article for his memorial publication, I chose the very important subject, from the point of view of man's nutrition, of the production of milk on a protein-free feed using urea and ammonium nitrogen as the sole sources of nitrogen, which for several years up to the present time has been intensively studied in our Laboratory. I dedicate this article to the memory of HANS VON EULER with whom I worked in the winter of 1923—1924 and who became after that my close friend.

ARTTURI I. VIRTANEN

One far-reaching question concerning the basis of milk production has for some years been one of the main objects of study at the Biochemical Research Institute, Helsinki, that is the production of milk using a protein-free feed, with urea and ammonium salts as the sole sources of nitrogen. Experiments in 1958 showed that when cows were fed ammonium nitrogen labelled with $^{15}N$ all the amino acids of the milk protein became labelled; in other words all had been synthesised from the ammonium nitrogen ([1]) [2])). The labelling of some essential amino acids, particularly histidine, was, however, much weaker than that of others. It followed from this result that it should be possible to develop by progressive adaptation a rumen microbial population capable, to a greater degree than normal, of using ammonium nitrogen for protein synthesis. In this way it could be expected that the cow could synthesise, by means

TABLE I.

Feeding experiments with $(^{15}NH_4)_2SO_4$ or $CO(^{15}NH_2)_2$, test cow Eiru (MOISIO, KREULA and VIRTANEN)

| | Test cow Eiru, calved 11-2-'63, exp. feeding 25 months $(^{15}NH_4)_2SO_4$ fed 12-4-'64 milk obtained 3 h after feeding | | Test cow Eiru, calved 3-10-'62, exp. feeding 6 months $CO(^{15}NH_2)$ fed 20-10-'62 milk obtained 6.3 h after feeding | | Test cow Hieno, normal feeding $(^{15}NH_4)_2SO_4$ fed 14-5-'58 milk obtained 15 h after feeding | |
|---|---|---|---|---|---|---|
| | 6.3 h | | 20 h | | 25 h | |
| Tot.-N | 0.036 (71) | 0.069 (82) | 0.080 (82) | 0.102 (91) | | |
| Prot.-N | 34 (67) | 69 (82) | 78 (80) | 108 (96) | 0.039 (72) | 0.032 (107) |
| Rest-N | 87 (171) | 88 (105) | 42 (42) | 61 (55) | | |
| Amide-N | 93 (182) | 128 (152) | 130 (133) | 100 (89) | 70 (130) | 39 (130) |
| Glu | 51 (100) | 84 (100) | 98 (100) | 112 (100) | 54 (100) | 30 (100) |
| Asp | | 77 (92) | 90 (92) | 125 (112) | 47 (87) | 37 (123) |
| Tyr | 26 (51) | 61 (73) | 72 (74) | 123 (110) | 42 (78) | 36 (120) |
| Ala | 31 (60) | 70 (83) | 81 (83) | 110 (98) | 47 (87) | 38 (127) |
| Ser | | 64 (76) | 81 (83) | 104 (93) | | |
| Threo | | 43 (51) | 55 (56) | 113 (101) | 38 (70) | 31 (103) |
| Gly | 39 (76) | 45 (54) | 89 (91) | 102 (91) | 44 (82) | 33 (110) |
| Vol | 19 (37) | 52 (62) | 58 (59) | 110 (98) | 32 (59) | 32 (107) |
| Pro | 22 (43) | 56 (67) | 65 (66) | 109 (97) | 26 (48) | 23 (77) |
| Lys | 23 (45) | 64 (76) | 75 (77) | 132 (118) | 29 (54) | 31 (103) |
| Met | 19 (37) | 47 (56) | 73 (74) | 126 (112) | | |
| Leu | 22 (43) | 52 (62) | 56 (58) | 101 (90) | 33 (61) | 29 (97) |
| Ileu | 32 (63) | 68 (81) | 68 (70) | 100 (89) | | |
| His | 14 (28) | 36 (43) | 38 (40) | 83 (74) | 8 (15) | 9 (30) |
| Phe | 24 (47) | 48 (57) | 58 (59) | 87 (78) | 27 (50) | 27 (90) |
| Arg | 23 (45) | 57 (68) | 59 (60) | 98 (88) | 22 (41) | 22 (73) |

The numbers in parenthesis show the labelling of different amino acids and other N-fractions as per cent of the labelling of glutamic acid.

of the microorganisms in its rumen, an amount of protein (and at the same time of essential amino acids) sufficient not only for the maintenance of the animal, but also for the production at least of a small amount of milk. Feeding experiments, which to date have been in progress for more than three years, have shown for the first time that this indeed is the case ([2-8]). By using purified, protein-free feed (starch, cellulose and sucrose) and urea and ammonium salts as the sole sources of nitrogen surprisingly high milk yields have been obtained. The highest annual yield so far has been about 4200 kg milk calculated as 4% fat-containing standard milk. The labelling of the essential amino acids was greatly increased on the test feeding (Table I).

The composition of the milk — we call it zero milk (0-milk) — produced on protein-free feed has been studied from many sides in our laboratory. The composition of the milk proteins has naturally been the object of great interest and in this article some of our results in this field are presented.

## Experimental

### Preparation of the sample

The milk samples which were to be studied were received from different parts of southern Finland. In the special cow house on Prof. VIRTANEN's farm Joensuu, 27 km from Helsinki, the test cows Aino, Eiru, Jairu and Metta were fed purified carbohydrates (cellulose, starch and sucrose), urea and ammonium nitrogen forming the sole source of nitrogen. As control samples were used the milk of the herd (26—30 Ayrshire cows) of the same farm (control milk I), the feed being principally AIV-silage, crushed oats and smaller amounts hay during the winter and pasture in summer. Last winter some urea was added to the feed. The proteins of the milk of cows on different normal winter feed have been studied too. The main feed of the Ayrshire cattle of the Rintala farm contained clover-timothy-hay, crushed oats, malted barley from brewery, minerals (300 g) and oat straw. The total milk of the cattle of the farm house (control milk II) and the milk of separate cows Ella, Rape and Heta were taken as samples for the protein studies. The feeding of the cattle of the Ersta farm (31 Ayrshire cows) consists of clover-timothy-hay, AIV-silage made of clover-timothy, crushed oats, protein-rich concentrates, mixture, molasses, feeding chalk and oat straw. The mixed milk from this farm (control milk III) has been studied as well as the milk of separate cows Jetta, Hento and Hyöty. The milk samples of the Joensuu farm were each time taken from the evening and morning milk (1:1). The milk samples of the Ersta and the Rintala farms were taken from the morning milk.

The milk samples were cooled after milking, warmed in the labora-

tory to 39—40° C and separated three times. The separated skim milk was cooled to 1—2° C and dialyzed for 48 hours against deionized water agitated with a magnetic stirrer and changed 4 times. The casein and whey protein fractions were prepared from another portion of the original skim milk. The casein was precipitated by acidifying the sample with 1N HCl to pH = 4.6 and standing overnight at 1—2° C at this pH and then centrifuging. After thorough washing with deionized water, the casein was dissolved in dilute NaOH and reprecipitated with acid. After further washing the casein preparation was then dialyzed by the procedure similar to the above. The acid whey from the casein precipitation was adjusted to pH = 7.0 with dilute sodium hydroxide and traces of casein were reprecipitated at pH = 4.6. The whey was centrifuged, adjusted to pH = 6.7 and dialyzed. After dialyzing the samples were freeze-dried.

### Nitrogen determination

Nitrogen determinations were made by the standard micro-KJELDAHL procedure (A.O.A.C. 38.009—38.011).

### Amino acid analysis

The milk total protein, casein, whey and the fractions separated on the Sephadex column were hydrolyzed with 6 N HCl at 106° C and the amino acids determined with the automatic amino acid analyzer made in our laboratory.

### Sephadex gel filtration

A LKB 4900 A ReCyChrom column, which is designed for analytical separation mainly by the technique of gel filtration was used. The recycling experiments were made according to the method of PORATH and BENNICH[9]) on a 3.6 × 90 cm bed of Sephadex G-100 equilibrated with a buffer solution consisting of 0.1 M Tris and 0.5 M sodium chloride adjusted with HCl to pH = 8.0, and on a bed of Sephadex G-200 equilibrated with a buffer solution consisting of 0.05 M phosphate buffer and 0.25 M sodium chloride, pH = 6.5. The bottom and top parts of the column are similarly slightly conical, having small channels connected to capillary tubing. The sample can be applied to the bottom of the column, eluted upwards with a peristaltic pump and withdrawn from the system through a special valve during the experiment. A LKB Uvicord flow analyzer operating at 253 mμ and a recorder were connected to the column. The elution rate was 12 ml per hour. The fractions obtained by gel filtration were collected, dialyzed, concentrated and subjected to polyacrylamide gel electrophoresis.

## Paper electrophoresis

Paper electrophoresis was performed by the technique of SCHULTE and MÜLLER[10]). Whatman 1, Whatman 3 MM and Schleicher & Schüll 2043 a were used as supporting media. Electrophoresis was performed at pH 8.6, the most satisfactory buffer for this being 0.1 M Michaelis veronal buffer, and at pH = 8.9, 0.5 M Tris — 0.021 M ethylenediaminetetraacetic acid (EDTA) — 0.075 M boric acid. Paper electrophoresis at high voltage was carried out with Pherograph original Frankfurt D. B. G. M. and at low voltages with the Elphor Standard Chamber. The paper strips were fixed and stained with saturated Amido Black in methanol solution to which 10% glacial acetic acid was added. Methanol-acetic acid solution was used for decolorizing.

## Starch gel electrophoresis

The electrophoretic separation was performed by the vertical method described by SMITHIES[11]), and WAKE and BALDWIN[12]), with minor modifications. To prepare the gel 46 g of "Starch-Hydrolyzed" (Connaught Medical Research Laboratories, Toronto; 1 to 191-1) was suspended in a conical flask in 240 ml Tris-citrate buffer solution (0.76 M Tris and 0.005 citric acid) according to POULIC[13]). The final volume was 370 ml. The suspension was mixed continuously and heated over a flame until the semisolid mass became a viscous liquid. At this stage solid urea was added slowly and dissolved by continuous stirring and heating to a final concentration of 7 M. Air bubbles were removed very quickly by application of vacuum with a pump while avoiding the loss of water from the suspension. Of the side-chain interactions which are known to stabilize protein conformations, disulfide linkages are undoubtedly the most stable and important. To avoid these interactions, 0.2 ml mercaptoethanol was added for each 100 ml of buffer solution after evacuation of the hot gel solution. The clear viscous liquid was then poured into a perspex tray made in our laboratory according to SMITHIES[11]), with minor alterations. Three layers of Whatman 3MM paper were used to form the electrical contact between the electrode solution and the gel. Paper strips moistened in the bridge solution were set quite straight at both ends of the tray. The tray was covered as soon as possible with plexiglass, excluding air bubbles, with a comb with 8 teeth to dip into the starch gel. The gel was covered and allowed to set overnight at room temperature. Urea gels set slowly but are strong and water clear.

Following the setting of the gel the slotformer and the cover were carefully withdrawn and the samples were inserted with a thin-tipped pipette into the gel slots without any supporting material. Each slot was filled completely and then sealed with a thin piece of gel, excluding air bubbles. Then the gel was covered

with paraffin at a temperature not exceeding 55° C. The electrode vessels were filled with a bridge solution of 0.3 M boric acid titrated to pH = 8.6 with sodium hydroxide.

The vertical electrophoresis was carried out at 1° C. Electrical contact between the electrode solution and the gel was made by Whatman 3MM as straight as possible to ensure the formation of a sharp boundary which moves to the anode and at which there is the change in potential gradient. The running time of 17 hours was used for the separation of casein the voltage being 180, after which the brown boundary had moved about 12 cm from the starting slots.

After the electrophoretic run, paraffin was removed and the gel was sliced into two slices with a wire. The freshly cut surfaces of the gels were stained with a 1% Amido Black in methanol-water-acetic acid (50:50:10) solution and washed with the same solvent mixture without staining by the methods of SMITHIES. The gel can be preserved for some time in the same solution.

### Disc electrophoresis

The disc electrophoresis was carried out according to ORNSTEIN and DAVIS, except for minor modifications. The cylindrical glass tubes (0.5 × 6.5 cm) in a vertical position in the tube stand are filled with small pore solution having 1.5 cm from the top free. The solution in each tube is overlaid carefully with a few drops of distilled water by means of a syringe. A sharp boundary appeared at the interface. The tubes are left for 30 minutes at room temperature for the gels to form. All the stock solutions and working solutions are made according to ORNSTEIN and DAVIS[14]).

The gelation time is dependent on temperature and may be adjusted by the addition of potassium ferricyanide. Following gelling the water and unreacted monomer solution are removed completely and large-pore solution of the thick 7 mm is added. The water layer is overlaid of the gel solution as the above. The tubes are photopolymerized behind a fluorescent lamp 15—20 minutes. After photopolymerization of the spacer-gel, the water and unreacted monomer are decanted and the other large-pore solution with protein sample (150—250 μg) is added to each tube. The tubes are photopolymerized 30 minutes and inserted into the grommets of the upper reservoir. Both reservoirs are filled with Tris-glycine buffer previously diluted 1 : 10 with distilled water. One ml of 0.001% Bromphenol Blue is stirred into the upper buffer. Air spaces above and at the bottom of each gel tube are removed by filling with buffer.

The apparatus for electrophoretic running is prepared in our laboratory. It is made of perspex with platin electrodes and capable of running 12 samples at a time. In the electrophoretic running,

positive electrode on the bottom, the current is adjusted to 4.5 mAmp. per tube.

Electrophoresis is continued until the band of Bromphenol Blue dye has migrated 30 mm, which takes about 30—40 minutes. The running is carried out at the temperature of 1° C. At the completion of electrophoresis, the gels are removed from the tubes with a steel wire by rimming under water. Each gel is stained with 1% Amido Black in 7% acetic acid solution by heating 10 minutes. Electrophoretic destaining is carried out in the destaining tubes in the same apparatus in 1½—2 hour. The destained gels can be stored in 7% acetic acid several months with little alterations.

## Results and Discussion

### Amino acid analysis

The results of the amino acid analysis of the total protein of milk and of the casein are shown in Table II and of the whey in Table III. One can see no differences between the normal milk and the milk of the test cows. The amino acid composition of casein and total milk protein are very much similar to each other. Instead, differences are to be found between these and the amino acids of the whey. The amounts of asparagine, alanine, cysteine and lysine of the whey

TABLE II.

The amino acid composition of total protein and casein of 3 test cows and control milk produced on normal feeding.

| Amino acids found | Total protein | | | | Casein | | | |
|---|---|---|---|---|---|---|---|---|
| | Aino | Eiru | Jairu | Control milk | Aino | Eiru | Jairu | Control milk |
| Asp | 7,50 | 7,05 | 7,71 | 7,64 | 6,90 | 6,86 | 6,87 | 6,75 |
| Thr | 4,22 | 4,38 | 4,48 | 4,37 | 4,01 | 4,02 | 4,24 | 4,08 |
| Ser | 5,48 | 5,63 | 5,58 | 5,61 | 5,77 | 5,65 | 5,65 | 5,68 |
| Glu | 20,80 | 21,71 | 20,50 | 21,63 | 21,69 | 22,04 | 21,84 | 21,74 |
| Pro | 9,44 | 9,41 | 9,19 | 9,24 | 10,10 | 10,10 | 10,08 | 10,27 |
| Gly | 1,93 | 1,88 | 1,80 | 1,87 | 1,80 | 1,77 | 1,66 | 1,72 |
| Ala | 3,41 | 3,25 | 3,38 | 3,18 | 2,90 | 2,84 | 2,92 | 2,87 |
| Cys | 0,67 | 0,74 | 0,79 | 0,69 | 0,48 | 0,36 | 0,46 | 0,43 |
| Val | 5,49 | 5,50 | 5,55 | 5,43 | 5,66 | 5,55 | 5,60 | 5,68 |
| Met | 2,48 | 2,69 | 2,46 | 2,61 | 2,85 | 2,83 | 2,78 | 2,85 |
| Ileu | 4,35 | 4,33 | 4,54 | 4,31 | 4,34 | 4,33 | 4,41 | 4,40 |
| Leu | 9,23 | 9,33 | 9,05 | 9,03 | 8,71 | 8,48 | 8,39 | 8,60 |
| Tyr | 4,71 | 4,82 | 4,89 | 4,76 | 5,53 | 5,44 | 5,57 | 5,46 |
| Phe | 4,62 | 4,52 | 4,46 | 4,45 | 4,80 | 4,73 | 4,71 | 4,78 |
| Lys | 7,52 | 7,30 | 7,76 | 7,33 | 6,91 | 7,16 | 7,27 | 7,20 |
| His | 2,53 | 2,52 | 2,56 | 2,50 | 2,61 | 2,68 | 2,58 | 2,60 |
| Arg | 3,35 | 3,13 | 3,35 | 3,21 | 3,30 | 3,50 | 3,33 | 3,27 |
| $NH_3$ | 2,27 | 1,79 | 1,92 | 2,15 | 1,63 | 1,61 | 1,63 | 1,62 |

are clearly greater than those of casein and total protein. Glutamic acid, proline and leucine content are lower in the whey. Quantitative differences as to the amino acids of the whey proteins between various cows also occur to a greater extent than in the amino acids of casein and total milk. The fact that the whey proteins are denaturated easier than casein when isolating protein from the milk, may also have some influence on this.

TABLE III.

The amino acid composition of whey proteins of four test cows and control milk produced on normal feeding.

| Amino acids found | Whey proteins | | | | |
|---|---|---|---|---|---|
| | Aino | Eiru | Jairu | Metta | Control milk |
| Asp | 10,00 | 10,87 | 10,67 | 10,70 | 10,69 |
| Thr | 5,02 | 5,06 | 5,59 | 5,21 | 5,16 |
| Ser | 4,66 | 4,41 | 5,33 | 4,69 | 4,86 |
| Glu | 16,97 | 16,87 | 16,70 | 17,24 | 17,63 |
| Pro | 5,49 | 5,32 | 5,38 | 5,40 | 5,50 |
| Gly | 2,13 | 2,05 | 2,22 | 2,37 | 2,07 |
| Ala | 5,08 | 4,86 | 4,75 | 5,31 | 4,57 |
| Cys | 2,54 | 2,90 | 2,45 | 2,52 | 2,25 |
| Val | 5,49 | 5,44 | 5,85 | 5,48 | 5,53 |
| Met | 2,12 | 1,78 | 1,99 | 2,26 | 1,88 |
| Ileu | 5,21 | 5,43 | 5,20 | 5,50 | 5,41 |
| Leu | 11,79 | 12,10 | 11,45 | 12,01 | 11,44 |
| Tyr | 3,11 | 3,51 | 3,17 | 2,94 | 2,81 |
| Phe | 3,91 | 3,83 | 3,93 | 3,79 | 3,86 |
| Lys | 9,48 | 9,48 | 8,93 | 8,85 | 9,28 |
| His | 2,04 | 2,35 | 2,17 | 1,97 | 2,18 |
| Arg | 3,39 | 2,58 | 2,95 | 2,57 | 2,85 |
| $NH_3$ | 1,56 | 1,15 | 1,27 | 1,17 | 2,02 |

## The starch gel and paper electrophoresis of the casein

Starch gel is of great usefulness for the separating of the casein owing to its high resolving power. The gel probably acts as a molecular sieve, small molecules penetrating the swollen gel grains more easily than particles of larger size (SMITHIES[11]). Gels can be made in the presence of high concentration of urea. The resolving power of starch gel medium seems to be enhanged with urea addition. We have found that without urea casein is aggregated into the gel and one cannot see any separated bands. Concentrated solutions of urea disperse the casein components and usually prevent the aggregation (WAKE and BALDWIN[12]). Casein components are resolved into sharp bands in the urea-gel with the exception of k-casein. The spreading of k-casein is prevented by reducing

the disulfide linkages with mercaptoethanol (MACKINLEY and WAKE[15])). After that, k-casein can be resolved into several bands.

Vertical positioning of the tray during electrophoresis results in better resolution in the separation of casein components than the horizontal one. In the latter, gravity may cause accumulation of protein in the lower starch layers leading to uneven mobilities at different levels in the gel. Furthermore, the electroosmotic flow may counteract the electrophoretic migration.

Starch gel analysis of whole caseins of different cows are shown in Figures 1—4. Caseins are resolved into several bands, which move toward the positive electrode. NEELIN et al.[16]) have designated 21 zones, labelled in Fig. 1 and 2 according to these forms. They have identified the following zones:

| Zone no. | Component |
|---|---|
| 1 | λ-casein |
| 2— 4 | Unknown |
| 5— 8 | Major components of calcium-sensitive α-casein ($\alpha_s$ casein) |

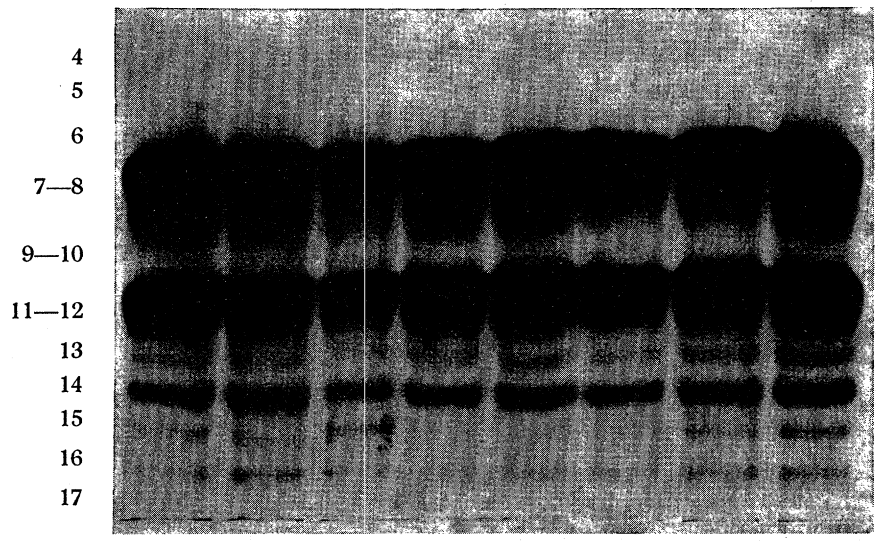


**Fig. 1.** Starch gel electrophoresis of acid caseins from milks of the test cows Aino (A), Metta (M), Jairu (J), Eiru (E) and control milk I ($C_I$).

The samples were taken in December 1964. Zone designations are according to NEELIN[18]).

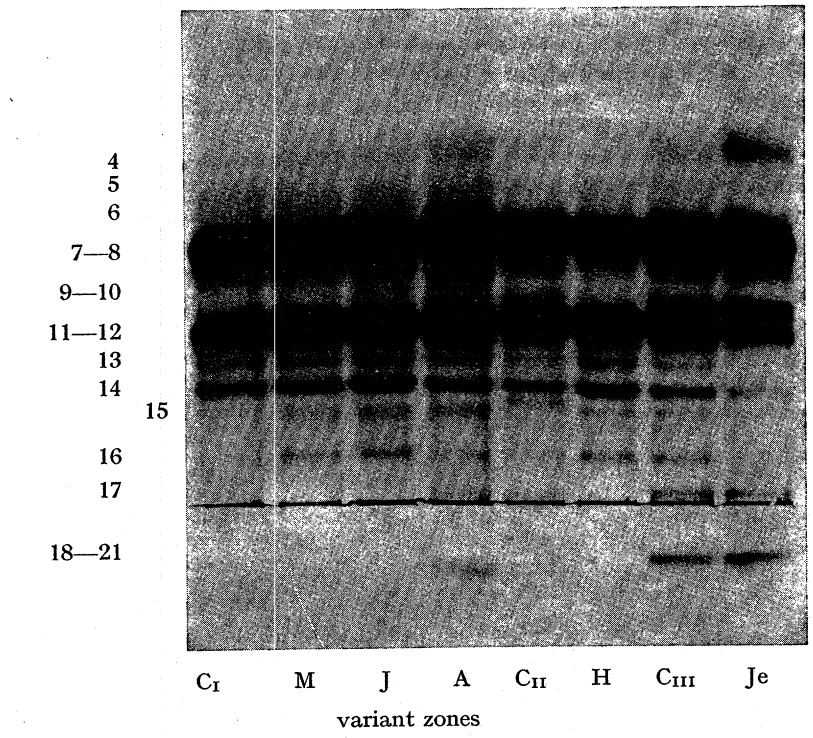


**Fig. 2.** Patterns of starch gel electrophoresis of caseins of individual cows. O-milk of Aino (A), Jairu (J), Metta (M) and control milk ($C_I$) from the Joensuu farm, Heta (H) and control milk II ($C_{II}$) from the Rintala farm, Jetta (Je) and control milk III ($C_{III}$) from the Ersta farm.

| | |
|---|---|
| 9—10 | Unknown, contaminants of both $\alpha_s$- and β-casein |
| 11—12 | Major components of β-casein |
| 13—15 | k-casein (especially Zone 14) |
| 16 | γ-casein (occasionally not distinct from Zone 17) |
| 17 | Unknown, probably a denatured fraction |
| 18—21 | Unknown, cationic zones |

We can see that the caseins of the individual cows in Figure 1 are quite similar. The numbers of the casein zones are the same in the milk of test cows and control cows. Resolution of individual cows caseins (Fig. 2) shows differences in the k-casein fraction, which is genetically variable (WOYCHIC[17])). The number of zones distinguished in our patterns is more than the total reported by NEELIN[18]). So especially in the region of k-casein. In the region of zones 13-15 there are found 3 (Fig. 1) or 4—5 (Fig. 2) different bands, which may give some evidence of genetic heterogeneity. The fraction (in the

region zone 15) of k-casein has the fast electrophoretic moving in control milk II; in control milk III and in milk of Heta it is divided clearly into two bands; Aino, Jairu, Metta and control milk I have a little thicker zone. The β-casein of Jetta is divided into two major components. In Fig. 2 it is observed that some zones are moving toward the cathode. The caseins are dissolved in these experiments 24 h before electrophoretic running into urea-Tris-citrate buffer containing mercaptoethanol, while the caseins in other experiments are dissolved into the same solvent only four hours before. May be that cationic zones are due to that.

The photographs are rather dark, so that it is impossible to see all minor components. In order to see the minor components, higher concentrations of sample should be used (Fig. 1), but the bands of major components are then spreading and run together. The zones at the top have moved slower than those in the middle part, which may result from the ununiformity of electrical field in the gel. The sharpening of the zones and the mobilities are temperature-dependent. The electrophoresis carried out at low temperature allows the shortening of the time of electrophoresis and the reducing of the spreading of zones caused by diffusion because of the increased voltage gradients. Heat development is markedly uniform throughout the gel, but because of the greater evaporation from the surface the temperature is greater within the gel than on surface. Distorted zones may be the result of temperature gradients and uneveness of the gel. The plastic cover on the tray during gelling tends to wrap so that the gel has not the same thickness in every place. This may produce the distortion of the applied electrical field.

Different proteins do not take up the same amount of dye for equal weights of protein so we cannot assume that the relative amounts of the components are proportioned on the gel. We can, however, correlate the relationship of the amount of the protein fractions of different cows' milk. Quantitative differences are observed between control milks and milks of individual cows.

By paper electrophoresis, the casein is resolved into four bands. Casein is not molecularly dispersed, but contains large aggregates, so absorption by the filter paper occurs and the zones are diffused. Starch gel is observed to be better than paper as electrophoretic medium in resolution of casein.

## Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed by ORNSTEIN and DAVIS for analytical separation of proteins. Polyacrylamide gels are synthetic polymers of two monomers of low molecular weight. This gel separates protein both according to charge and size. The pore size of polyacrylamide gels can be varied by adjustment of the monomer concentration and can be selected

depending on the dimensions of the material to be fractionated. Gels have good physical properties for use as an electrophoretic medium. They are thermostable, transparent, strong, relatively inert chemically, have few or no ionic side groups and a high resolving power. An analytical column is composed of 3 layers: 1. a large-pore gel containing the sample ions in which electrophoretic concentration of these ions is initiated, 2. a large-pore gel in which the concentration of the sample ions into a narrow zone prior to electrophoretic separation takes place, and 3. a small-pore gel in which electrophoretic separation takes place.

It is desirable to reduce the running time to a minimum to avoid diffusion and to achieve a good separation of the constituent ions. The thickness of the gel slab is limited to avoid the heating that occurs during an electrophoretic run when relatively high voltage gradients are used to shorten the time of fractionation.

The concentration is produced by introducing the mixture of sample ions into an electrophoretic column near the boundary of two ions. The leading ion has a mobility higher than either the proteins or the trailing ion of which the latter is chosen to have a lower mobility than the proteins. Application of a voltage results in the leading ions moving downwards and overtaking the proteins which are sandwiched into highly concentrated discs between the slow and fast ions before they move into the small-pore gel.

A drawing of the disc electrophoretic patterns of the whey proteins of individual and control cows are shown in Figs. 3 and 4. Disc electrophoreses of the proteins of the milk of the test cows

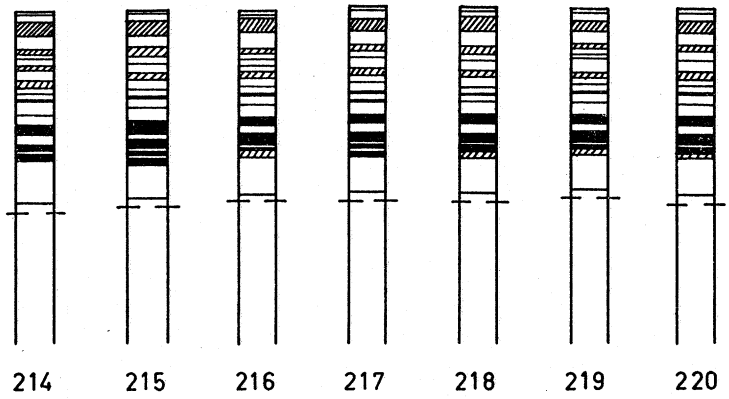


**Fig. 3.** Drawings of disc electrophoretic patterns of the whey proteins of the test cows, Aino (214, 218), Jairu (215), Metta (216, 219) and control milk I (217, 220).
The samples in tubes 214—217 were taken in February and ni tubes 218—220 in April 1965.

Aino, Jairu and Metta, of separate cows fed in different ways, and of the whey proteins of the control milk are shown in the figures. 100 mg of protein sample was dissolved in diluted Tris-HCl buffer (pH = 6.7) used in preparing the spacer gel, and 200 μg of the solution was taken. The solution contained also 5% sucrose. Increased density facilitates subsequent overlayering, so the sample must have a higher density than the eluant.

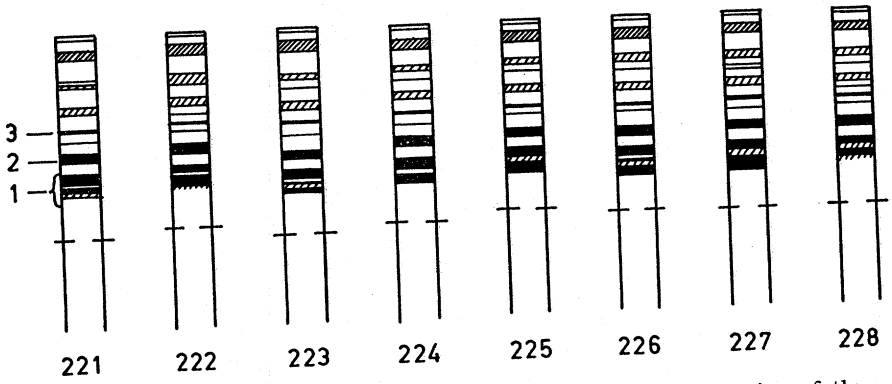


**Fig. 4.** Drawings of electrophoretic patterns of the whey proteins of the individual cows, which are fed in different ways.
Ella (221), Heta (222), Rape (223) and control milk II (224) were taken from the Rintala farm, control milk III (225), Hento (226), Hyöty (227) and Jetta (228) were taken from the Ersta farm. The numbers identify the main components of whey proteins: 1. β-lactoglobulins; 2. α-lactalbumin; 3. serum albumin.

When comparing the whey proteins of the milk of cows on different feeds after separation by disc electrophoresis, only very small differences were observed (Figs. 3 and 4). The discovery of the genetic variants of whey proteins and caseins is characterized. The genetics of β-lactoglobulins A and B have been published by ASCHAFFENBURG and DREWRY[19]) and β-lactoglobulin C by BELL[20]). These genetic variants have been concerned with proteins which differ in electrophoretic mobility. From figures 3 and 4 it can be seen that β-lactoglobulin is sometimes resolved into 2 zones, and sometimes into 3. β-lactoglobulin was identified by concurrent disc electrophoresis of both whey proteins and a pure BDH preparation manufactured according to ASCHAFFENBURG and DREWRY[19]) in which 3 zones and slight impurities of serum albumins and immunoglobulins can be found. These 3 successive zones are identical with the corresponding zones of Jairu, for example. The slowest moving of these three zones is to be found in every whey, assuming that the line formed by bromophenol blue has moved exactly the same

distance during the electrophoresis. While making several simultaneous runs, the mobility of bromophenol blue is not the same in every tube. Then the electrophoresis must be interrupted at different times to remove the tubes which are ready. This may cause slight discrepancies between different samples.

Of the whey proteins of the test cows, β-lactoglobulin of Jairu was always resolved into 3 zones, and that of Aino and Metta into 2. The β-lactoglobulins of the milk of the cows Rape and Hento on a feed of hay/malted barley or hay/AIV silage and of the control milk I corresponded to the zones of Jairu, Ella and Heta, and those of Jetta corresponded to the zones of Aino. The β-lactoglobulins of the control milks II and III as well as those of the cow Hyöty were also resolved into two zones, but the electrophoretic mobility of the slowest moving of these zones corresponded to that of the middle zone of Jairu. The β-lactoglobulin zones of the control milk are not identical either in these experiments during different months, but sometimes there are 2 and sometimes 3 zones. This is possibly due to genetic variations in the separate cows.

Immunoglobulin fractions nearest to the starting zone containing antibodes are easily diffusing to such a degree that it is difficult to determine separate zones exactly. In the disc electrophoresis of the whey proteins of the colostrum, we have noticed that the amount of immunoglobulins is very great and is decreased after 10—12 milkings to normal. The serum albumin fraction which is identified by running serum and the whey samples side by side is identical in all wheys. The same is the case with the third main component which appears between the serum albumin and β-lactoglobulin and which according to GROVES[21] is an α-lactalbumin. The unidentified fraction moving immediately behind the zone of bromophenol blue appears only in some samples. The gels were cut after the electrophoresis run from the bromophenol line, and it is possible that in electrophoretic colour-removing this fraction is mixed just with the blue bromophenol line moving afore.

## The gel filtration

The gel filtration is a fractionation procedure in which the separation depends to a large extent on differences in molecular size. The gel particles consist of a three-dimensional network with pores which prevent large molecules entering, but allow the penetration of small molecules. The greater the size difference, the greater is the difference in elution rate. The whey proteins of the test cows and control cows are fractionated on gel filtration. With Sephadex G-100, whey proteins are fractionated into 5 components on every cow and with Sephadex G-200 into 6 components. The fractions are collected, dialyzed, concentrated and subjected to PAGE electrophoresis. In electrophoretic patterns one can see very many

zones from every peak. This indicate that Sephadex does not fractionate skim milk proteins into electrophoretically distinct components, but into fractions of proteins having similar particle size. Disc electrophoretic patterns from every peak of test cows and control cows are quite similar.

## Summary

The milk proteins of cows on different feeding have been studied using gel filtration and electrophoretic methods in which paper, starch gel or polyacrylamide gel have been supporting media. When urea and ammonium nitrogen are the sole sources of nitrogen in the feeding of the cows, no greater differences can be found in the protein fractions of the milk samples than can be seen in the milk of cows on normal feed. The differences may depend on genetic variations which have recently been found in β-lactoglobulins and in the different casein fractions. Differences in amino acid composition of the proteins of test and normal milk have not been found.

## Zusammenfassung

Die Milchproteine verschieden gefütterter Kühe sind mittels Gel Filtration und Elektrophorese (wobei Papier-, Stärke- oder Polyacrylamidgel als stützende Media gedient haben) untersucht worden. Wenn Harnstoff und Ammoniumsalze die einzigen Stickstoffquellen im Futter der Kühe waren, konnten keine grösseren Unterschiede zwischen den Proteinfraktionen dieser Milch (O-Milch) und denen von Milch normalgefütterter Kühe gefunden werden. Die vorhandenen kleinen Unterschiede können auf genetischen Variationen beruhen, wie sie in der letzten Zeit in ß-Lactoglobulinen und in den verschiedenen Caseinfraktionen verschiedener Kühe gefunden worden sind. Es wurde festgestellt, dass Unterschiede in der Aminosäurezusammensetzung des Total-Proteins und Caseins der O- und der Normalmilch analytisch kaum nachweisbar sind.

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